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Effect of sodium butyrate on performance, immune status, microarchitecture of small intestinal mucosa and lymphoid organs in broiler chickens

Arbab Sikandar[1,*], Hafsa Zaneb[2,*], Muhammad Younus[1], Saima Masood[2], Asim Aslam[2], Farina Khattak[3], Saima Ashraf[2], Muhammad Shahbaz Yousaf[2], and Habib Rehman[2]

* **Corresponding Authors:** Arbab Sikandar
Tel: +92-334-4038376, **Fax:** +92-47-7671262;
E-mail: arbab.sikandar@uvas.edu.pk
Hafsa Zaneb
Tel: +92-42-9921-1449 (238),
E-mail: hafsa.zaneb@uvas.edu.pk

[1] Sub-campus, Jhang, University of Veterinary and Animal Sciences, Lahore 35200, Pakistan
[2] University of Veterinary and Animal Sciences, Lahore 54000, Pakistan
[3] Monogastric Science Research Centre, Scotland's Rural College, Ayr KA6 5HW, UK

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Objective: This study aimed to examine the effect of sodium butyrate (SB) on growth performance, immune status, organs weights, and microarchitecture of lymphoid organs and small intestine.
Methods: A total of 120, 1-d-old broiler chicks were distributed into the following four treatment groups: corn-soy based basal diet (BD) without supplement (control), or the same BD supplemented with 0.1 g/kg zinc bacitracin (ZnB), 0.5 g/kg SB (SB-0.5), or 1.0 g/kg SB (SB-1), respectively. Six birds/group were killed on d-21 and d-35, and samples were collected.
Results: Cell-mediated immune response at 48 h post-Phytohemagglutinin-P injection, and antibody titer against Newcastle disease vaccine and sheep red blood cells on d-35 was noted higher ($p<0.05$) in SB-1 compared to ZnB and control. Lower ($p<0.05$) feed conversion ratio (FCR) was attained by the supplemented groups. Thymus and spleen weighed more ($p<0.05$) in SB-1, and bursa registered more ($p<0.05$) weight in both SB groups compared to control. On d-21, areas of thymus medulla and spleen germinal centers were noted higher ($p<0.05$) in SB-1 group. The villus height and villus surface area increased ($p<0.05$) in duodenum and jejunum in both SB groups on d-21, and in SB-1 on d-35, respectively compared to ZnB and control. On d-21, number of goblet cells containing mucins of acidic nature increased ($p<0.05$) in all the segments of small intestines in SB-1 group compared to control, and on d-35 in ileum compared to other groups.
Conclusion: In conclusion, SB improved growth performance and immunity as well as modulated morphology of lymphoid organs and gut mucosa in broiler chickens.

Keywords: Organic Acid; Poultry; Antibiotic Growth Promoter; Mucosa; Intraepithelial Lymphocytes; Goblet Cells

INTRODUCTION

After imposition of ban on using some dietary antibiotics as growth enhancers by the European Union [1], the focused area in the poultry and animal sector is to find/create safe feed items which are free of antibiotics [2]. Subsequently, the research focused on development of optimal alternatives with the aim to maintain functions of gut and immune system. Organic acids (commonly known as acidifiers) and their salts are generally considered as harmless and have been approved by most technologically advanced countries to be used as a feed additive for animals. The acidifiers, including sodium butyrate (SB) is known for decreasing the gut mucosal pH, thus creating an acidic environment for the growth of normal commensals [3]. They overcome the development and proliferation of some *Salmonella* spp. [4]. There were noteworthy improvements in weight gain, carcass characteristics and increase in size of intestinal villi in the butyric acid-supplemented

birds [5]. Chamba et al [6] used SB in broilers and observed its growth promoter effect. Enriched IgG concentration in serum and total IgA+ in jejunum was observed in SB treated piglets [7], and the immunostimultory property of SB has also been highlighted in chicken [8] by inducing host defense peptides. Hence the SB was registered as an immune modulator [9]. It has been observed that the microencapsulated (coated with fatty acid matrix) type of organic acid was more effective than an antibiotic growth promoter (Enramycin) in rising growth performance in broilers [10]. The microencapsulated butyrate delivered portion of the butyrate to be free further distal in the intestinal tract because of slow release during digestion [4] and causes mucosal modulation in the gut [6]. Its use led to a tendency towards better growth performance, lower colonization and fecal shedding of *Salmonella* compared to the non-protected feed supplements [4]. To- date, limited reports have been published to evaluate the effects of SB on humoral and cellular immune status [9] and microarchitecture of visceral organs, including the segments of small intestine, bursa of Fabricius, thymus and spleen in broiler chickens. A comprehensive study was, therefore, needed to assess such effects in commercial chickens.

The aim of the current study was to evaluate whether the gut development and immune system of broiler chickens is influenced by feeding microencapsulated SB from day-1.

MATERIALS AND METHODS

Experimental chicks, husbandry and ration

A total of 120 1-d-old M77 Hubbard broiler chicks (as hatched) were reared for a total period of 35 days in environmentally controlled shed, where the temperature and relative humidity (RH) were maintained at 32°C±1°C and 70%±5%, respectively at the beginning. The temperature was reduced by 3°C per week till it reached 24°C±2°C, with RH closed to 65%±5%; thereafter, the temperature was maintained till d-35. Broiler chicks at the age of d-1 were weighed and divided into the following four groups: corn-soy based basal diet (BD) with no supplement (control), BD supplemented with10% zinc bacitracin (ZnB, Hubei Yuancheng Tech. Develop. Co. Ltd., Wuhan, China added 0.01% of feed) or 1.0 g/kg SB (SB-1) or 0.5 g/kg SB (SB-0.5), respectively. Each group had 30 birds with three replicates (n = 10) each. The birds in each group were offered a BD prepared locally in feed mill without antibiotics (Table 1) in starter (d-1 to d-21) and grower (d-22 to d-35) phases, respectively. Feed was formulated accordingly to meet the energy and nutrient requirements of broilers and was supplemented with SB (CM3000, Hangzhou King Techina Feed Company Ltd., Hangzhou 311112, China) as described elsewhere [11] with the following minor modification. The microencapsulated SB was weighed and was thoroughly homogenized with 1 kg mesh form of BD. To ensure its proper mixing, the resulting mixture was then mixed in vertical mixer with the control mesh form of BD. Availability of diet and water was *ad libitum* throughout the study. Six broiler chickens/group were killed on d-21 and d-35, and samples including blood and organs were collected.

Table 1. Control diet ingredients and calculated analysis

Items	Starter phase	Grower phase
Ingredient (%)		
Corn	40.15	57.57
Rice broken	15.0	-
Soy meal	11.54	9.60
Sunflower meal	12.00	13.00
Canola meal	9.00	5.00
Rapeseed meal	5.00	7.60
Rice polish	-	4.00
Guar meal	1.00	-
Wheat bran	1.34	-
Molasses	2.00	-
Sodium bicarbonate	0.03	0.065
Sodium chloride	0.21	0.21
Di-calcium Phosphate	1.33	1.49
L-lysine	0.30	0.35
DL-Methionine	0.10	0.12
Vit-mineral premix[1)]	1.00	1.00
Nutrient composition		
Calculated ME (kcal/kg)	2,750	2,850
CP (%)	19.6	18.5
DM (%)	87.0	88
Crude fiber (%)	6.05	6.35
Crude fat (%)	2.16	2.35
Total ash (%)	5.77	5.40

[1)] Vitamin mineral premix (each kg contained): ascorbic acid, 26,000 IU; retinol, 200,000 IU; cholecalciferol, 80,000 IU; tocopherol, 1,072 IU; thiamine, 11,666 IU; pyridoxine, 33,333 IU; menadione, 11,333 IU; riboflavin, 54,000 IU ; niacin, 5,36,000 IU; folic acid, 13,600 IU; methylcobalamin, 223 IU; biotin, 1,340 IU; Ca, 195 g; K, 70 g; Na, 18 g; Mg, 6 g; Fe, 2,000 mg; Zn, 1,200 mg; Mn, 1,200 mg; Cu, 400 mg; I, 40 mg; Co, 20 mg; and Se, 8 mg.

Growth performance

The average body weight (ABW), weekly weight gain (WWG), average feed intake (AFI) and FCR were studied to record weekly growth performance [12].

Immune response evaluation

Three *in-vivo* tests in which cutaneous response to phytohemagglutinin-P (PHA-P) and serum antibody level produced in response to Newcastle disease vaccine and sheep red blood cells (SRBCs) were measured to evaluate the immune competence in broiler chickens treated with SB.

Cell-mediated immunity

Cell-mediated immunity was assessed through injection of PHA-P (Sigma-Aldrich, St. Louis, MO, USA) as reported by Corrier et al [13] with the following slight modification. Briefly, two birds per replicate (n = 6 per group) were randomly selected on d-17 and the PHA-P solution (prepared in sterile phosphate-buffered

saline [PBS]) was injected intradermally (100 μg/100 μL/chicken) between the 3rd and 4th digits of the right foot. The left foot served as control and was injected with 100 μL of PBS. The net increase in thickness of the injected sites was evaluated on 24, 48, and 72 hours post-injection using pressure sensitive micrometer. The immune response (foot web index) to PHA-P was measured by subtracting the left foot thickness from that of the right foot.

Humoral immunity

The antibody titer or antibody production level is attributed to humoral immune response in animals. Humoral immune responses against Newcastle disease virus (NDV) and sheep RBCs antigens were evaluated through serological titration as defined previously [14]. Briefly, all the chicks were vaccinated with ND vaccine (Nobilis ND LaSota, Intervet International B.V. Boxmeer, Holland) on d-1 and d-9 via the ocular route and boosted on d-16 and d-23 via drinking water. And in case of SRBCs, 2 birds per treatment replicate (n = 6 per group) were randomly selected, wing banded and injected bilaterally with 5% SRBCs antigen (sheep blood collected in Alsevier's solution, washed thrice and suspended in phosphate buffer saline) in two parts (0.5 mL each, intramuscularly in both sides of the *Musculus pectoralis*) on d-14. Booster dose was given on d-21. Blood samples were collected on seven days post-primary injection and on d-35. Sera separated (2,000×g for 10 minutes) and were stored at –20°C till analysis. The antibody responses to NDV and SRBCs were measured using micro-titer hemagglutination inhibition and hemagglutination (HA) assays, respectively as mentioned by King [14].

Relative weight of lymphoid organs

Two chickens per treatment replicate were randomly selected, weighed and killed on d-21 and 35. Small intestine, liver, spleen, thymus and bursa of Fabricius were isolated from the carcass and their relative weights were evaluated. Representative samples of the organs were then subsequently collected in 10% neutral buffered formalin for histological processing.

Microarchitecture of immune organs

Thymus: Histomorphometry of the thymus was carried out as described Madej et al [15] with slight modifications; briefly, the thymic lobules were split into 4 sections by two lines crossing each other at right angle in the center of the medulla. All the lines expressing overall widths of the cortex were evaluated and the average was represented as cortical thickness. Area of the medulla was evaluated by uniting the measurements of two lines expressing the length and width of the medulla. Afterward, the ratios of cortex to medulla were evaluated in three well-oriented lobules per section, and the mean values obtained from three sections per bird were calculated.

Spleen: Well-oriented germinal center areas in the spleen were combined together and were noted as a percentage of the total field of view at 10× [15]. Later the average of three sections values was determined.

Bursa: Total numbers of lymphoid follicles in one microscopic field were recorded. Length, width and area of 5 well-oriented bursal follicles per section (3 sections per sample at 4×) was measured [16]. Later, average value of the three sections was noted.

Microarchitecture of small intestinal mucosa

Representative samples of approximately 2 cm (length) segments were excised from duodenum (10 cm distal to the junction between duodenum and gizzard), jejunum (5 cm proximal to the Meckel's diverticulum) and ileum (5 cm proximal to the ileo-cecal junction). Intestinal samples were processed using paraffin embedding technique, sectioned at 5 μm using a microtome (AMOS Scientific AEM-450, St. Veit/Glan, Austria), and stained with H&E [17] similar to the immue organs, except for histochemical differentiation of goblet cells for which the slides were stained using combined alcian blue periodic acid-Schiff technique [18].

Gut mucosal histomorphometry: Three sections were collected (one section after every 10 sections) from each intestinal sample. From each section, five well-orientated villi having intact lamina propria were selected randomly for examination. Consequently an average of 15 values was analyzed for each sample. Finally, the mean values from six chickens were noted as mean values for one treatment. Slides were examined under a light microscope (Olympus CX31, Olympus, Hicksville, New York, USA) at 4× magnification, supported with digitalized live image analysis program (Olympus DP20, Olympus, USA). The variables calculated for histomorphological modulations were villus height, VH; villus width, VW; villus surface area, VSA; crypt depth, CD; villus height to crypts depth ratio, VH:C D [19, 12].

Intraepithelial lymphocytes count: Sections already used for morphometry were also used for intraepithelial lymphocytes (IELs) count as described previously by Ashraf et al [19]. The IELs are described as small (7 to 10 μm) and large lymphocytes, (10 to 20 μm) having intensely stained round to oval nucleus surrounded by tiny cytoplasm and is positioned along the columnar epithelium [20].

Goblet cell histochemistry: Slides prepared and processed earlier were subjected to alcian blue periodic acid Shiff (AB-PAS) staining. Three sections were obtained from each intestinal segment and goblet cells were counted in 5 villi/section. Thus an average of 15 values was calculated for each sample. The histochemical differentiation on the basis of acidic and mixed (acidic and neutral) mucins was noted according to the methods described elsewhere by Ashraf et al [19]. Goblet cells containing acidic mucin were stained blue by the AB, whereas mixed mucin were stained purple by PAS staining.

Statistical analysis

The normal distribution of the data was confirmed using Kolmogorov-Smirnov test and data were presented as means±standard

error of the mean. The data were analyzed using one-way analysis of variance (SPSS for windows version 20, Chicago, IL, USA). Statistical differences among treatment means were determined through Duncan's multiple range tests. In all statistical analyses, p<0.05 was considered significant.

Ethical note

This research was approved by the Ethical Review Committee for the use of laboratory animals of the University (reference no.: DR/257 dated 13-04-15).

RESULTS

Growth performance

During the first week, the feed intake decreased (p<0.05) in SB-1 chicks compared to control and FCR was noted to be lower (p< 0.05) in ZnB compared to SB-0.5 and control groups. The ABW and WWG in the 4th week, and ABW in 5th week were noted to be greater (p<0.05) in the treated groups compared to control (Table 2). During 5th week the WWG increased (p<0.05) in SB-1 compared to control, and AFI was higher (p<0.05) in both SB-offered groups compared to ZnB and control. The collective average of WWG per week increased (p<0.05), and that of FCR decreased (p<0.05) in all the supplemented groups compared to control. The AFI per week was higher (p<0.05) in both SB-offered groups compared to ZnB and control.

Immunological responses

No significant difference was noted between mean values of different groups at 24 and 72 h. However, higher (p<0.05) skin thickness was observed in SB-1 at 48 h post PHA-P injection compared to ZnB and control groups (Table 3).

Immune responses on day-21 were non-significant among the groups; however, the SB-1 group registered a tendency towards

Table 2. Effect of Sodium butyrate and zinc bacitracin on performance in broilers

Growth phase	Parameters	Treatments				p-value
		SB-1	SB-0.5	ZnB	Control	
1st week	Avg. body weight (g)	151.13±3.45	148.67±1.43	151.40±1.31	152.03±1.13	0.545
	Weekly weight gain (g)	110.13±3.45	107.00±1.43	110.40±1.31	111.03±1.13	0.545
	Avg. feed intake (g)	130.87±2.30^{b}	129.57±1.34^{ab}	127.27±1.19^{ab}	134.10±5.06^{a}	0.042
	FCR	1.18±0.02^{ab}	1.21±0.02^{a}	1.15±0.03^{b}	1.20±0.02^{a}	0.092
2nd week	Avg. body weight (g)	420.50±4.59	422.76±7.73	412.37±2.02	410.43±3.81	0.296
	Weekly weight gain (g)	269.37±5.64	274.77±8.29	260.96±0.84	258.40±2.91	0.181
	Avg. feed intake (g)	340.73±8.79	341.30±1.14	348.70±1.01	350.23±0.98	0.369
	FCR	1.27±0.06	1.24±0.04	1.34±0.01	1.36±0.02	0.172
3rd week	Avg. body weight (g)	755.43±4.14	736.66±11.21	735.50±3.62	729.00±4.04	0.095
	Weekly weight gain (g)	334.93±8.50	313.90±12.66	323.13±5.21	318.56±7.71	0.433
	Avg. feed intake (g)	521.73±10.76	512.83±8.87	513.93±1.19	523.57±5.06	0.674
	FCR	1.56±0.03	1.64±0.08	1.58±0.03	1.64±0.02	0.532
4th week	Avg. body weight (g)	1,191.25±4.65^{a}	1,156.04±8.40^{a}	1,138.20±6.55^{a}	1,039.21±10.43^{b}	0.003
	Weekly weight gain (g)	435.82±8.21^{a}	419.37±6.10^{a}	402.71±8.11^{a}	310.21±10.78^{b}	0.001
	Avg. feed intake (g)	749.17±11.51	744.49±4.46	741.17±6.12	733.62±3.95	0.513
	FCR	1.72±0.00^{b}	1.78±0.07^{b}	1.84±0.02^{b}	2.37±0.09^{a}	0.001
5th week	Avg. body weight (g)	1,741.83±16.69^{a}	1,689.42±34.54^{a}	1,672.21±14.36^{a}	1,485.88±19.83^{b}	0.000
	Weekly weight gain (g)	550.58±16.78^{a}	533.38±43.52^{ab}	534.00±11.38^{ab}	446.67±28.81^{b}	0.107
	Avg. feed intake (g)	1,153.17±5.88^{a}	1,165.54±2.58^{a}	1,062.38±2.67^{b}	1,039.46±4.78^{c}	0.000
	FCR	2.09±0.06	2.21±0.18	1.99±0.04	2.35±0.17	0.301

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg); ZnB, zinc bacitracin 0.01% of feed; FCR, feed conversion ratio.
Means within a row marked with different superscripts were significantly different (p<0.05).
Values represent mean±standard error of the mean.

Table 3. Effect of sodium butyrate and zinc bacitracin on cell mediated immune response against PHA-P in broilers

Time interval post injection (h)	Skin thickness (mm) after PHA-P injection in various groups				p-value
	SB-1	SB-0.5	ZnB	Control	
24	0.71±0.04	0.66±0.04	0.65±0.04	0.57±0.05	0.209
48	0.65±0.02^{a}	0.55±0.03^{ab}	0.51±0.03^{b}	0.49±0.06^{b}	0.029
72	0.48±0.05	0.45±0.05	0.39±0.02	0.38±0.04	0.314

PHA-P, phytohemagglutinin-P; SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.
Means within a row marked with different superscripts were significantly different (p<0.05).
Values represent mean±standard error of the mean.

better response compared to other groups. On day-35, antibody titer against ND and SRBCs registered higher (p<0.05) in SB-1 compared with ZnB and control (Table 4).

Relative organ's weight

Bursa weighed more (p<0.05) in both SB-offered groups, while thymus weighed more (p<0.05) in SB-1 group compared to control on d-21 (Table 5). On d-35, spleen registered more (p<0.05) weight in SB-1, while thymus weighed more (p<0.05) in all the supplemented groups compared to control.

Histomorphometry of immune organs

On d-21 thickness of thymus medulla increased (p<0.05) in SB-1 compared to other groups. Germinal center area of spleen in SB-1 increased (p<0.05) compared to ZnB and control (Table 6). On d-35 germinal center area in spleen was greater (p<0.05) in both SB-offered groups compared to ZnB and control (Table 7).

Mucosal histomorphometry of small intestine

Starter phase (D-21): On day-21, VH and VSA in duodenum and jejunum of both SB-offered groups and VH in ileum of SB-1 group chickens was found enhanced (p<0.05) compared to ZnB and control. The VH:CD was increased (p<0.05) in duodenum and ileum of SB-1 group, and in jejunum of both SB groups compared to ZnB and control (Table 8).

Table 4. Effect of sodium butyrate and zinc bacitracin on humoral immune response in broilers

Treatments	Antibody titre (log_2) on various days			
	NDV		SRBC	
	Day-21	Day-35	Day-21	Day-35
SB-1	2.19±0.12	2.31±0.08^{a}	6.00±0.37	8.67±0.42^{a}
SB-0.5	2.16±0.07	2.21±0.07^{ab}	5.50±0.22	7.17±0.37^{ab}
ZnB	2.09±0.12	1.98±0.09^{b}	5.66±0.33	7.16±0.30^{bc}
Control	1.98±0.11	1.98±0.09^{b}	5.16±0.30	6.00±0.36^{c}
p-value	0.290	0.026	0.322	0.000

NDV, Newcastle disease virus; SRBC, sheep red blood cells; SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.
Means within a column marked with different superscripts were significantly different (p<0.05).
Values represent mean±standard error of the mean.

Grower phase (D-35): SB-1 group showed increased (p<0.05) VH and VSA in duodenum and jejunum compared to ZnB and control, and increased (p<0.05) VH and VSA in ileum compared to control. Villus width and VH:CD was higher (p<0.05) in duodenum of SB-1 compared to control (Table 9).

Intraepithelial lymphocytes count: The IEL count did not vary statistically among groups (Table 10).

Goblet cell histochemistry: On d-21, number of goblet cells con-

Table 5. Effect of sodium butyrate and zinc bacitracin on the relative organs weight in broilers

Treatments	Relative organs weight[1)]							
	At day-21				At day-35			
	Liver	Spleen	Thymus	Bursa	Liver	Spleen	Thymus	Bursa
SB-1	2.2272±0.19	0.1027±0.01	0.6206±0.03^{a}	0.3125±0.03^{a}	1.7085±0.02	0.1646±0.02^{a}	0.3861±0.00^{a}	0.1667±0.02
SB-0.5	2.1582±0.15	0.0974±0.02	0.5895±0.03^{ab}	0.2807±0.03^{a}	1.6320±0.05	0.1418±0.00^{ab}	0.3721±0.00^{a}	0.1550±0.02
ZnB	2.1210±0.14	0.0953±0.03	0.5221±0.02^{ab}	0.2700±0.04^{ab}	1.5971±0.05	0.1435±0.02^{ab}	0.3857±0.00^{a}	0.1617±0.02
Control	1.8735±0.09	0.078±0.01	0.5479±0.04^{b}	0.1804±0.02^{b}	1.5753±0.05	0.0960±0.01^{b}	0.3184±0.01^{b}	0.1167±0.02
p-value	0.395	0.199	0.146	0.044	0.172	0.065	0.000	0.295

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.
[1)] Relative organs weight = organ weight/body weight×100.
Means within a column marked with different superscripts were significantly different (p<0.05).
Mean±standard error of the mean.

Table 6. Effect of sodium butyrate and zinc bacitracin on immune organ morphology of broilers during starter phase

Organs	Parameters	Treatments				p-value
		SB-1	SB-0.5	ZnB	Control	
Thymus	Thymic cortex (μm)	308.33±24.71	292.33±18.65	295.83±12.24	288.17±11.49	0.865
	Thymic medulla (μm^2)	604.50±21.13^{a}	522.00±17.02^{b}	523.67±26.29^{b}	494.00±25.26^{b}	0.016
	Cortex:medulla	0.5050±.03	0.5633±.04	0.5741±.04	0.5912±.04	0.464
Spleen	Germinal center/field area (%)	0.7636±0.02^{a}	0.7361±0.01^{ab}	0.6465±0.04^{b}	0.6449± 0.03^{b}	0.032
Bursa	Bursal follicular length (μm)	508.33±61.15	500.67±21.78	485.50±29.20	468.67±44.10	0.791
	Bursal follicular width (μm)	161.17±7.53	151.17±9.55	155.50±8.29	142.67±8.75	0.493
	Bursal follicular area (μm^2)	81,648.6± 3562.2	75,901.3± 6365.3	75,004.3± 5042.7	66,691± 7330.1	0.354
	Bursal follicular number	9.50±0.43	8.33±0.42	8.83±0.48	8.67±0.71	0.470

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.
Means within a row marked with different superscripts were significantly different (p<0.05).
Values represent mean±standard error of the mean.

Table 7. Effect of sodium butyrate and zinc bacitracin on immune organ morphology in broilers during grower phase

Organs	Parameters	Treatments				p-value
		SB-1	SB-0.5	ZnB	Control	
Thymus	Thymic cortex (μm)	271.83 ± 12.84	242.67 ± 13.29	257.67 ± 12.96	234.83 ± 17.91	0.303
	Thymic medulla (μm^2)	432.50 ± 26.39	398.83 ± 18.66	404.67 ± 27.33	364.50 ± 14.18	0.234
	Cortex:medulla	0.6406 ± 0.05	0.6151 ± 0.04	0.6473 ± 0.04	0.6449 ± 0.04	0.950
Spleen	Germinal center/field area (%)	0.8876 ± 0.08^a	0.8619 ± 0.08^a	0.7550 ± 0.05^{ab}	0.5329 ± 0.09^b	0.018
Bursa	Bursal follicular length (μm)	398.50 ± 23.37	389.50 ± 12.48	373.67 ± 21.53	373.17 ± 17.56	0.730
	Bursal follicular width (μm)	142.83 ± 13.79	124.50 ± 15.89	134.50 ± 18.24	104.67 ± 9.97	0.691
	Bursal follicular area (μm^2)	56,326.3 ± 5,067.4	47,595.5 ± 4,839.2	50,285.0 ± 7,721.8	38,022.5 ± 3,932.6	0.509
	Bursal follicular number	8.17 ± 0.98	7.17 ± 1.83	7.50 ± 0.67	7.00 ± 0.58	0.559

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.
Means within a row marked with different superscripts were significantly different ($p < 0.05$).
Values represent mean ± standard error of the mean.

Table 8. Effect of sodium butyrate and zinc bacitracin on small intestine morphology of broilers on d-21

Intestinal segments	Parameters	Treatments				p-value
		SB-1	SB-0.5	ZnB	Control	
Duodenum	Villus height (μm)	1,321.12 ± 29.17^a	1,316.29 ± 28.89^a	1,019.86 ± 17.35^b	1,033.25 ± 14.83^b	0.000
	Villus width (μm)	138.76 ± 7.73	133.20 ± 9.52	138.76 ± 17.27	119.62 ± 6.49	0.521
	Villus surface area (mm^2)	0.578 ± 0.04^a	0.552 ± 0.05^a	0.453 ± 0.05^{ab}	0.388 ± 0.02^b	0.016
	Crypt depth (μm)	136.50 ± 6.07^{ab}	143.67 ± 3.13^a	118.30 ± 2.52^c	126.58 ± 4.19^{bc}	0.002
	VH:CD	9.73 ± 0.24^a	9.17 ± 0.18^{ab}	8.63 ± 0.18b^c	8.21 ± 0.33^c	0.002
Jejunum	Villus height (μm)	928.17 ± 16.73^a	899.00 ± 18.73^a	686.00 ± 10.77^b	658.00 ± 17.64^b	0.000
	Villus width (μm)	105.37 ± 3.63	105.61 ± 4.17	107.17 ± 4.29	107.53 ± 1.94	0.935
	Villus surface area (mm^2)	0.307 ± 0.09^a	0.297 ± 0.08^a	0.232 ± 0.07^b	0.222 ± 0.04^b	0.000
	Crypt depth (μm)	106.13 ± 2.42	106.55 ± 3.79	104.07 ± 6.35	106.68 ± 2.75	0.960
	VH:CD	8.77 ± 0.30^a	8.47 ± 0.22^a	6.76 ± 0.56^b	6.19 ± 0.22^b	0.000
Ileum	Villus height (μm)	494.10 ± 19.55^a	474.08 ± 18.07^{ab}	422.03 ± 16.43^{bc}	417.20 ± 17.87^c	0.015
	Villus width (μm)	104.93 ± 2.50	104.87 ± 2.75	102.48 ± 2.19	99.82 ± 3.33	0.513
	Villus surface area (mm^2)	0.163 ± 0.09^a	0.156 ± 0.082^{ab}	0.136 ± 0.06^b	0.131 ± 0.09^b	0.031
	Crypt depth (μm)	112.30 ± 2.56	111.05 ± 3.11	109.88 ± 3.75	110.93 ± 2.99	0.959
	VH:CD	4.40 ± 0.16^a	4.27 ± 0.14^{ab}	3.85 ± 0.17^{ab}	3.79 ± 0.26^b	0.083

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed; VH, villus height; CD, crypt depth.
Means within a row marked with different superscripts were significantly different ($p < 0.05$).
Values represent mean ± standard error of the mean.

taining acidic mucins was greater ($p<0.05$) in all the segments of small intestine in SB-1 group compered to control (Table 11). On d-35, the acidic-natured goblet cells increased ($p<0.05$) in ileum of SB-1, compared to all other treatment groups (Table 12).

DISCUSSION

The growth performance of SB-1 group was numerically better compared to control group (Table 2), linked well with the observations of Chamba et al [6] and Dehghani-Tafti and Jahanian [21]. The variability in FCR between control and the treatment groups is due to unidentified factors, however, it is assumed that better performance may be due to the creation of the acidic environment in the gut after SB consumption [3], which in turns minimizes the load of pathogens [10]. Average weekly feed intake was noted higher ($p<0.05$) in both SB supplemented groups compared to ZnB and control. Zinc bacitracin used in the current study because this antibiotic is famous for having growth promoting properties and are being practiced in local poultry sector. The infeed SB may improve the intraluminal digestibility of mineral and proteins which may result in improved weight gain in SB offered groups as mentioned by Zhang et al [22].

Scientists take interest in using *in-vivo* T cell dependent immune function test to PHA-P [23], and antibody response to heterologous erythrocytes [24]. For immune-competence of commercial chicken fed diet containing SB, we used these tests. T-cell mitogenic PHA-P was injected intradermally to evaluate the cell-mediated immunity. We found the net increase in swelling of the PHA-P injected area in SB-1 at 48 h compared to ZnB and control groups (Table 3), which indicates better immune response in that group [25]. The cell mediated immune response might be due to delayed type of hypersensitivity. Sheep RBCs (SRBCs) act as Thymus-dependent immunogens and are used for antibody response evaluation in chickens [24]. We observed higher

Table 9. Effect of sodium butyrate and zinc bacitracin on small intestine morphology in broilers on d-35

Intestinal segments	Parameters	Treatments				p-value
		SB-1	SB-0.5	ZnB	Control	
Duodenum	Villus height (μm)	1,470.62±33.99[a]	1,290.63±17.22[b]	1,116.70±27.02[bc]	1,102.41±44.49[c]	0.000
	Villus width (μm)	180.44±6.39[a]	159.87±5.39[ab]	151.60±19.34[ab]	129.62±6.48[b]	0.030
	Villus surface area (mm^2)	0.832±0.03[a]	0.607±0.02[b]	0.532±0.07[bc]	0.449±0.03[c]	0.000
	Crypt depth (μm)	137.92±9.49	140.49±9.20	113.30±2.20	119.85±12.93	0.174
	VH:CD	10.87±0.64[a]	9.18±0.59[b]	9.87±0.25[ab]	9.34±0.59[b]	0.015
Jejunum	Villus height (μm)	835.06±43.73[a]	772.19±2495[ab]	643.30±30.77[c]	685.15±14.61[bc]	0.001
	Villus width (μm)	127.12±6.41[a]	103.22±3.91[b]	112.85±4.64[ab]	110.70±5.69[b]	0.031
	Villus surface area (mm^2)	0.3362±0.03[a]	0.2493±0.01[b]	0.2280±0.01[b]	0.2387±0.01[b]	0.003
	Crypt depth (μm)	130.55±3.94	128.41±3.04	121.79±5.02	124.76±9.62	0.689
	VH:CD	6.39±0.27	6.03±0.27	5.37±0.46	5.75±0.52	0.330
Ileum	Villus height (μm)	460.67±14.63[a]	451.86±6.96[ab]	436.17±8.09[ab]	429.44±5.66[b]	0.112
	Villus width (μm)	114.34±3.01	107.37±6.01	104.85±7.14	102.39±5.18	0.477
	Villus surface area (mm^2)	0.1652±0.01[a]	0.1520±0.01[ab]	0.1432±0.01[ab]	0.1383±0.01[b]	0.111
	Crypt depth (μm)	105.22±2.26	111.05±2.69	105.36±3.18	104.78±1.94	0.264
	VH:CD	4.38±0.09	4.08±0.08	4.16±0.14	4.10±0.09	0.203

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed; VH, villus height; CD, crypt depth.
Means within a row marked with different superscripts were significantly different ($p<0.05$).
Values represent mean±standard error of the mean.

Table 10. Effect of sodium butyrate and zinc bacitracin on intraepithelial lymphocytes count

Treatments	Day					
	21			35		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
SB-1	41.17±2.26	44.33±1.67	45.83±2.17	68.83±3.07	56.17±2.25	56.17±2.06
SB-0.5	43.83±1.64	40.83±2.21	45.67±1.41	66.50±1.67	46.83±4.06	51.50±2.87
ZnB	43.66±2.84	47.33±1.52	44.33±2.15	65.83±2.65	57.16±3.62	60.50±2.53
Control	43.00±4.52	39.50±5.56	42.00±1.93	63.66±6.99	49.33±4.49	48.66±4.30
p-value	0.919	0.327	0.491	0.850	0.187	0.060

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.
Means within a column marked with different superscripts were significantly different ($p<0.05$).
Values represent mean±standard error of the mean.

Table 11. Effect of sodium butyrate and zinc bacitracin on differentiated goblet cells count in small intestine of broilers on d-21

Intestinal segments	Goblet cell mucin type	Treatments				p-value
		SB-1	SB-0.5	ZnB	Control	
Duodenum	Acidic	47.50±2.94[a]	44.00±3.88[ab]	40.50±4.22[ab]	34.67±1.16[b]	0.066
	Mixed	38.00±3.88	38.67±4.51	41.00±3.62	42.17±3.37	0.857
	Total	85.50±4.02	82.67±6.46	81.50±4.77	76.83±3.98	0.661
Jejunum	Acidic	50.67±3.60[a]	44.00±3.88[ab]	40.50±.4.22[ab]	36.33±.80[b]	0.047
	Mixed	38.83±4.66	38.67±4.50	41.00±3.62	42.16±3.47	0.912
	Total	89.50±4.48	82.67±6.46	81.50±4.77	78.50±3.89	0.474
Ileum	Acidic	45.00±2.73[a]	43.67±1.56[ab]	44.17±2.24[ab]	38.17±174[b]	0.126
	Mixed	39.17±2.36	37.83±1.64	38.83±2.73.62	41.83±2.55	0.669
	Total	82.83±2.44	82.83±3.96	83.00±2.52	80.00±4.06	0.903

Means within a row marked with different superscripts were significantly different ($p<0.05$).
Values represent mean±standard error of the mean.
SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.

($p<0.05$) titer results against ND and SRBCs in SB-1 group on day-35 (Table 4). This indicates that SB may modulate the function of B and T cells in later stages of the antigenic exposure and can regulate the host immunity [9,23]. Park et al [26] noted that short chain fatty acids including butyrates, are commonly synthesized in the gut which support the regulation and growth of Th1 and Th17 effector cells as well as interleukin-10 (IL-10) regulatory T-cells. These cells maintain the immune system framework.

Table 12. Effect of sodium butyrate and zinc bacitracin on differentiated goblet cells count in small intestine of broilers on d-35

Intestinal segments	Goblet cell mucin type	Treatments				p-value
		SB-1	SB-0.5	ZnB	Control	
Duodenum	Acidic	53.67 ± 1.81	53.83 ± 9.17	51.16 ± 3.55	47.83 ± 232	0.826
	Mixed	37.33 ± 2.17	38.83 ± 3.34	42.00 ± 4.13	45.16 ± 3.96	0.414
	Total	91.83 ± 3.43	92.67 ± 7.65	93.16 ± 4.41	93.00 ± 5.48	0.992
Jejunum	Acidic	51.33 ± 4.26	50.83 ± 5.54	44.00 ± 3.94	39.50 ± 1.94	0.161
	Mixed	42.33 ± 1.28	45.67 ± 3.68	42.67 ± 2.66	47.66 ± 2.61	0.465
	Total	93.67 ± 3.40	96.50 ± 4.19	86.67 ± 4.63	87.17 ± 4.35	0.284
Ileum	Acidic	53.17 ± 3.02[a]	45.67 ± 2.12[b]	43.50 ± 1.89[b]	39.17 ± 2.04[b]	0.003
	Mixed	32.83 ± 2.14	39.33 ± 2.89	35.33 ± 2.79	36.17 ± 2.12	0.356
	Total	86.00 ± 4.82	85.00 ± 4.02	78.83 ± 3.38	75.33 ± 1.15	0.149

SB-1, SB-0.5, sodium butyrate 1.0 g/kg, 0.5 g/kg; ZnB, zinc bacitracin 0.01% of feed.
Means within a row marked with different superscripts were significantly different ($p < 0.05$).
Values represent mean ± standard error of the mean.

The effect of butyrate on macrophage cell line was reported by Zhou et al [27]. They noted that butyrate inhibited nitric oxide production, and diminished the cytokines expression, including IL-6, IL-10, interferon gamma, and IL-1β, which controlled inflammation and maintained immune homeostasis. Similar to our findings, Eshak et al [28] reported high antibody titer against ND in SB treated chickens. The butyrate regulates the macrophage activities in intestine, and the macrophage effectuates the function of T cells and dendritic cells in the gut [29]. The latter cells also have a role in host immunity. The supplemented SB increased number of IgA+ cells which later on produced secretory immunoglobulin-A in piglets jejunum [7]. The sIgA contributes in improvement of one of the first line of mucosal defense. It was published that butyrate activated the immunomodulatory property through the production of host defense peptides [8] and has no effect on provoking inflammation [3].

In healthy animals the increase in weight of immune organs is correlated with improved immune responses of the body. Bursa, thymus and spleen are the key players of the immune system and their weights in the current study increased ($p<0.05$) in SB-offered chickens (Table 5). Alike our finding, Eshak et al [28] reported that bursa weighed more ($p<0.05$) in chickens treated with SB. The increased weights may be due to increased thickness of the parenchymal areas.

The greater sized thymus medulla and germinal center area in spleen in SB treated chickens (Table 6), and an increasing trend of bursal parameters observed in SB-1 group indicated that SB may effectuate the systemic immune systems in broilers also. To the best of our knowledge, no such study is available in literature highlighting the effect of SB on compartmental changes in immune organs to which we may compare our results.

Small intestine is the site for absorption in which the available nutrients are taken up through epithelial cells and drained into the general circulation. Architectural modulation of the small intestine is assumed to have a relationship with production performance of animals. We noted that small intestinal parameters including VH, VSA, and VH:CD in SB-offered groups improved significantly (Tables 8, 10). Butyrate acts as a rich source of energy for the enterocytes [9], and it may possibly increase the cell mitosis in the crypts. The SB may protect the mucosal epithelium from injury and alleviate the enteropathic stress [19] by increasing thyroid hormone in the circulation (data not reported). We found improved histomorphometrics in SB offered groups in duodenum and jejunum compared to ZnB and control. These findings proposed that the incoming ingesta containing SB at ileum had earlier been presented to utmost absorption in the former gut lumen and displayed better effect there.

The increased villus length and surface area could predict the gain in weight [19]. As the ingredient status of the diets in all groups was almost similar, the apparent enhancement in growth performance of the SB groups compared to control was assumed to be the result of the mucosal architectural modulations in those groups. Similar to our observation, various scientists reported that organic acid supplementation markedly increased the intestinal absorption area by promoting villus growth in height [5,11].

IELs are a mucosal portion of gut-associated lymphoid tissue and are expected to play important role in early contacts with antigens. Increased number of IEL is reported to have a role in immune modulations in infected animals offered *Enterococcus faecium* NCIMB 10415 [20]. In the current study the IELs population among groups was not dissimilar statistically (Table 10).

The mucus in the goblet cells acts as lubricant, source of nutrition for the normal commensals and protection of the gut from pathogens [30]. We found increased ($p<0.05$) acidic natured goblet cells in all the segments of small intestines in SB-1 group (Table 11). It is assumed that SB may uphold the goblet cells activities by regulating its mucin gene and may positively contribute to the protective mechanisms in the gut. On d-35 the underlying mechanism involved in increased acidic natured goblet cell population in the ileum is not clear; however, it may be in response to the high microbial population in that segment compared to the upper segments. In conclusion, diets supplemented with 1.0 g/kg SB stimulated a positive influence on the animal health by modulation of mucosal morphology of small

intestine in birds. These microarchitecture modulations have a relationship with improved immunity, which suggests that SB at 1 g/kg feed may be a proper replacement for the antibiotics.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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